Calverial cell Preparation

Reagents:

PBS: PBS (Ca, Mg free) + P/S

Culture media: □-MEM media (Gibco #12571-063) + L-glu + 10%FBS + P/S + 2-mercaptoethanol (2-ME) (Gibco BRL cat#21985-023 0.5ml→500ml)

Washing media: □-MEM media (Gibco #12571-063) + L-glu + 5%FBS + P/S

Digestion solution: 200u/ml collagenase (317U/mg) in PBS
Incubate at 370C for 20 minutes and sterile filter it.

4mM EDTA 0.5M EDTA pH 8.0 (Research genetics cat#750009): PBS = 1:125
0.4ml 0.5M EDTA in 50ml PBS. Sterile filter

Equipment:

Dish	100mm Petri dishes	
Water bath	37^0C	
50ml Erlenmeyer flasks	autoclave	
polypropylene mesh	200-297u	autoclave
Dissection instrument:	in 70% ethanol	
Paper tower	autoclave	
Pad	autoclave	
Pin	in 70% ethanol	

procedure:

1. Euthanize the mice on ice.
2. Dip the bodies into three successive ethanol tubes to sterilize them.
3. Cut off the head with scissors and fix the head on a dissecting board (covered with sterile paper tower) with a pin between the eyes
4. Insert a small scissors under the skin from the back of the head to the front, and move down on both sides. Cut open to reveal the calvaria with scissors on the right side and on the top right behind the pin (leave the left side attach)
5. Using very sharp spring scissors to make 5 cuts alone the edges of the calvaria:
    a. 1^{st} cut: on the bottom right cover
    b. 2^{nd} cut: on the right bottom edge of calvaria.
    c. 3^{rd} cut: on the left bottom edge of calvaria
    d. 4^{th} cut: on the right edge of calvaria.
    e. 5^{th} cut: on the top edge of calvaria
6. Flip the calvaria to the left side and cut it off. Put the calvaria into a 100mm petri dish containing washing media.
7. After all the calvaria have been dissected out, cut each calvaria into half and trim off the soft tissues and periosteum. Put all the clean calvaria into a new petri dish containing washing media.
8. Transfer all the calvaria to a 50ml Erlenmeyer flask containing 10ml 4mM EDTA and place it in the shaking (90 oscillations/min) water bath for 10 minutes.

9. **Carefully aspirate out the liquid, and rinse with 10ml PBS.**
10. **Add 10ml 4mM EDTA and place it in the shaking (90 oscillations/min) water bath for 10 minutes.**
11. **Carefully aspirate out the liquid, and rinse with 10ml PBS.**
12. **Aspirate PBS, add 5 ml collagenase solution into flask and place it in the shaking (90 oscillations/min) water bath for 10 minutes.**
13. **Pour the liquid into a 15ml Conical tube and centrifuge for 5 min at 1000rpm. Add 10ml PBS into flask, and then put the flask on ice.**
14. **After centrifuge, Aspirate the PBS from flask and transfer the cell-free collagenase from 15ml conical tube to flask. Place the flask in the shaking (90 oscillations/min) water bath for 10 minutes.**
15. **Carefully aspirate out the liquid, and rinse with 10ml PBS**
16. **Aspirate PBS, add 5 ml fresh collagenase solution into flask and place it in the shaking (90 oscillations/min) water bath for 15 minutes.**
17. **Carefully pour collagenase solution into a 50ml Conical tube through a mesh, remove the mesh. Rinse the calvaria with 10ml PBS, pour into the same tube through a mesh, add 5ml PBS with 5% FBS, and then put the tube on ice.**
18. **Repeat 16 and 17 twice.**
19. **Pour all the digest solution together, and then centrifuge the tube for 5min at 1000rpm.**
20. **Resuspend the pellets in 3ml culture media and count. Plate into tissue culture dishes.**
21. **Chang the media nest day, check cells every day for confluence. Write observations:**

22. **When the cells reach 80% confluency, freeze them.**

Freeze primary Calvariacells
Reagents:

Culture Media: □**-MEM media (Gibco #12571-063) + L-glu + 10%FBS + P/S + 2-mercaptoethanol (2-ME) (Gibco BRL cat#21985-023 0.5ml→500ml)**

PBS: **PBS w/o Ca, Mg**
Trypsin-EDTA: **(0.5%/0.2%): GIBCOBRL cat# 25300-054**
Frozen media: 80% FBS + 20% DMSO keep at 4^0C all the time

Put frozen box at refrigerator before start.

1. **Aspirate media**
2. **Wash 2X with PBS**
3 **Trypsin-EDTA: 15ml/150mm dish (For other sizes: divide plating volume by 2)**
4. **CO_2 incubator until cells round up (~ 2min)**
5. **Add ~15ml media, scrape the cells, transfer to a 50ml tube**
6. **Spin suspension for 5mins. at 1000 rpm, 4^0C**
7. **Re-suspend pellet in media, count**

(Keep cell suspension and frozen vials on ice)

8. **Add 0.5 ml cell suspension to each frozen vial, and then add 0.5 ml frozen media dropwisely to each vial with cells.**
9. **Put the vials into frozen box, and put the frozen box into –70^0C.**
10. **Next day, put vials into liquid nitrogen.**